PATENT

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APPLICATION FOR UNITED STATES LETTERS PATENT

for

ALPHA-DIFLUOROMETHYLORNITHINE (DFMO) SUPPRESSES POLYAMINE LEVELS IN THE HUMAN PROSTATE

by

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BACKGROUND OF THE INVENTION

This application claims priority to U.S. Provisional Application Ser. No. 60/227,714 filed August 24, 2000, herein incorporated by reference. The government may own rights in the present invention pursuant to grant number P30CA62203U19 from Public Health Service and CA81886 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services.

I. Field of the Invention

The present invention relates generally to the field of cancer therapy. More particularly, it concerns the use of DFMO (α-difluoromethylornithine), an irreversible enzyme inhibitor of the biosynthetic pathway of polyamines, in treating prostate cancer.

II. Description of Related Art

Because rapidly-proliferating cell-growth disease states are widespread throughout the world and affect a significant proportion of the population, they have been the subject of intensive research efforts. Unfortunately, despite such efforts and despite some successes, the overall control of these diseases has not been satisfactory. Recently, however, promising therapeutic methods for treatment of such disease states have been developed employing irreversible inhibitors of enzymes involved in the biosynthesis of the polyamines necessary for cell growth. Particularly useful enzyme inhibitors are those which produce *in vivo* irreversible inhibition of ornithine decarboxylase (ODC), the enzyme which catalyzes the decarboxylation of ornithine to putrescine.

α-Difluoromethylornithine (DFMO) is an irreversible inhibitor of ornithine decarboxylase (ODC) and causes depletion in the intracellular concentrations of putrescine and its derivative, spermidine (Pegg, 1988). Levels of spermine, which is derived from spermidine, are not as markedly affected by the enzyme inhibition. DFMO was initially synthesized for therapeutic anticancer usage, but it was found not to be an active cytotoxic agent in chemotherapy trials against human cancer (McCann and Pegg, 1992), except perhaps demonstrating moderate activity in the treatment of malignant

brain tumors (Levin *et al.*, 1987). In general, the compound was nontoxic, with the significant exception of hearing loss, which was reversible after the drug treatment was discontinued (Meyskens *et al.*, 1986). The onset of the hearing loss could be associated with total cumulative dose (Croghan *et al.*, 1991).

In experimental animal models, DFMO has been found to be a potent inhibitor of carcinogenesis that is especially active in preventing carcinogen-induced epithelial cancers of many organs, including those of the colon (Weeks *et al.*, 1982; Thompson *et al.*, 1985; Nowels *et al.*, 1986; Nigro *et al.*, 1987). DFMO acts late in the tumor-promotion phase in animals, but the precise mechanism by which it inhibits the development of polyps and cancers is unknown. Effects on cell transformation, invasion, and angiogenesis by ornithine decarboxylase and polyamines have been reported (Auvinen, 1997); for example, overexpression of ODC enhances cellular transformation and invasion (Kubota *et al.*, 1997).

Molecular mechanisms to explain this phenomenon include activation of mitogen-activated protein (MAP) kinase activity, secretion of matrix metalloproteinases (Wallon *et al.*, 1994; Kubota *et al.*, 1997) and pathways with oncongenes (c-*myc* and *ras*) (Meyskens *et al.*, 1999; Clifford *et al.*, 1995). A comprehensive review of the potential cellular interactions of polyamines has been recently presented (Auvinen, 1997).

Prostate cancer is the most commonly diagnosed malignancy in United States males, and is the second leading cause of male cancer deaths in the U.S. (Feuer *et al.*, 1999). As the incidence of prostate cancer increases with the age, the number of men who will be diagnosed with prostate cancer will increase as medical care and overall health improve. As such, the prevention of prostate cancer is of national medical concern. Current strategies for prostate cancer prevention have focused on changing the hormonal milieu in the prostate (Proscar), or adding antioxidants, (selenium, and vitamin E) or retinoids to the diet. Another approach is to suppress the polyamine levels in the prostate, an avenue suggested by studies indicating that ornithine decarboxylase (ODC), the first enzyme in the polyamine pathway, is over-expressed in human prostate cancer tissue (Mohan *et al.*, 1999).

ODC activity and polyamine content are higher in prostatic tissue when compared to other mammalian tissues (Dunzendorfer, 1978). In 1979, Danzin *et al.* treated rats with DFMO for two weeks and found maximum inhibition of prostatic ODC activity within 4-6 hours to 10% of baseline activity and sustained suppression to 50% of baseline activity throughout the study. The prostate was more sensitive to DFMO than the testis, thymus, spleen or liver, and the prostate and thymus showed a reduction in organ weight. Danzin *et al.* (1979) also looked at the effect of DFMO on prostatic regrowth with external testosterone after castration. After castration, the prostate decreased in size as did polyamine content. With androgen supplementation, prostatic atrophy was readily reversed, and polyamine content increased. DFMO markedly slowed prostatic (ventral lobe) weight gain during androgen treatment.

Heston *et al.* (1982) measured the suppressive effects of DFMO on prostate cancer cell lines *in vitro* and *in vivo* (after the inoculation of tumor cell into the flasks of rats to produce gross tumors). *In vitro* ODC activity was highest in the two androgen independent prostate cancer cell lines, followed by the ventral lobe, a slow growing cancer cell line and the dorsal lobe. *In vivo* prostate tumor bearing animals treated with DFMO intraperitoneally for 18 days developed tumors with half the wet weight of untreated controls, with no systemic toxicity. In another study, total polyamine suppression (treatment consisting of a polyamine-free diet, DFMO, a second inhibitor of polyamine oxidase, and intestinal tract decontamination) was evaluated on the growth of the MAT-LyLu prostate cancer cell line transplanted into the flanks of rats (Movlinoux *et al.*, 1991). In human subjects, reports of polyamine deprivation leading to decreased PSA titers in patients with prostate cancer was commented on by Kergozien (1996).

In 1999, Messing *et al.* published their results of two weeks of oral DFMO on human prostate polyamine levels. In their study men were randomized to receive either two weeks of DFMO 0.5gm/m^2 (n=15) or placebo (n=10) prior to radical prostatectomy or cystoprostatectomy. At the time of surgery, tissue for the polyamine analysis was taken after surgical removal of the prostate using *ex vivo* core biopsies. When palpable, suspected prostate cancer nodules were sampled. The number of prostate cores taken ranged from two to six per subject, but the majority of the cores did not show cancer

despite the researchers efforts to sample cancerous areas. ODC activity and polyamine levels were measured in the cores. In addition, each subject's plasma testosterone, serum PSA, and prostatic acid phosphatase levels were measured, as well as ODC activity from volar arm skin punch biopsies. Fourteen men completed two weeks of DFMO with no toxicity. Mean putrescine levels were statistically lower in the DFMO treated group (1.43 versus 1.95, p=0.03). Significantly, there were no differences found in ODC activity, spermidine and spermine levels measured in their study (Messing *et al.*, 1999).

In light of these results, it remains unclear that DFMO is useful in treating patients with prostate cancer, especially with of the lack of any effect on spermine levels.

SUMMARY OF THE INVENTION

Thus, the present invention contemplates a method of decreasing spermine and/or spermidine levels in a human prostate cell comprising administering to said cell DFMO in an amount and duration sufficient to decrease the levels in said cell. The prostate has high levels of polyamines when compared to other organs. It also differs from other organs in the amount and in the percent reduction of spermine upon exposure to DFMO using the methods of the present invention.

In a preferred embodiment, the human prostate cell is a cancer cell, a non-cancerous cell or a benign hyperplastic cell. The cell may be in a patient during the administering of DFMO, and the DFMO employed may be substantially free of the L-enantiomer, or a be enriched in D-enantiomer relative to a racemic 50/50 mixture of D- and L-isomers.

In a preferred embodiment, the DFMO will be administered for about 3, 4, 5, 6, 8, 10 or 12 weeks. The duration of DFMO administration may also be longer and continue for 2, 3, 4, 5, 6, 8, 10 or more than 12 months. The duration of DFMO administration may be 2, 3, 4, 5, 10, 15, 20, 25, 30, 40 or 50 years, or may be for the life of the patient. In a preferred embodiment, the amount of DFMO administered is about 0.1 to 2.0 $gm/m^2/day$, or more preferred, about 0.5 $gm/m^2/day$. The amount of DFMO may be

about 0.1 to 2 g/day, or more preferred 0.25 - 1.5 g/day, or even more preferred 0.5 - 1.0 g/day. The DFMO may be administered *via* any common route. In a preferred embodiment, the DFMO is administered orally.

In preferred embodiments the spermine levels are reduced. This reduction is preferentially about 10%, 20%, 30%, 40%, 50%, 60%, 70% or 80% as compared to the spermidine levels in said cell prior to said treatment. In a preferred embodiment, the spermidine levels are reduced. This reduction is preferentially about 30%, 45%, 55%, 65%, 75%, 85%, 95% or 99% as compared to the spermidine levels in said cell prior to said treatment. In another preferred embodiment, the spermidine/spermine ratio of said cell also is decreased. In yet another embodiment, the putrecine level is reduce about 50%, 60%, 70%, 80%, 90%, 95% or 99%.

The present invention also concerns a method of treating a human subject afflicted with prostate cancer which comprises administering DFMO to the subject in an amount and duration sufficient to reduce spermine levels, the spermidine levels, or the spermidine/spermine ratio in prostate cells of said subject.

Yet another embodiment comprises a second therapy, which can include singly or in combination: reducing dihydroxytestosterone, administering dietary antioxidants such as selenium, vitamin E or both, administering retinoids, prostatectomy, a low polyamine diet, inhibiting polyamine oxidase, radiation or a hormonal therapy such as luperon, zoledex, fultamide or casadex.

In a further embodiment, the effectiveness of the DFMO therapy according to the present invention can be determined in the treatment of prostate cancer by diagnostic methods including, but is not limited to, analysis of prostate specific antigen (PSA), a prostate biopsy, a rectal exam, or analysis of PSA and rectal exam.

Other embodiments include methods for inhibiting development of prostate cancer in a subject at risk, inhibiting prostate cancer metastasis in a subject with primary prostate cancer, inhibiting prostate cancer progression in subjects having Stage 1 or Stage 2 prostate cancer, rendering a unresectable prostate cancer tumor resectable, and

inhibiting growth of a prostate cancer tumor. These methods comprise administering DFMO to a human subject in an amount and duration sufficient to reduce spermine and/or spermidine levels in prostate cells of said subject.

Also within the scope of the invention is a method of treating benign prostate hyperplasia in a human subject afflicted with benign prostate hyperplasia comprising administering DFMO to said subject in an amount and duration sufficient to stabilize or reduce the amount of polyamine produced by the hyperplastic cells, wherein said polyamine is spermine, spermidine or a combination of spermine and spermidine. The levels of prostate specific antigen (PSA) produced by the hyperplastic cells could also be stabilized or reduced upon treatment with DFMO.

A further embodiment of the invention includes a method for treating benign prostatic hyperplasia in a human subject afflicted with benign prostatic hyperplasia comprising administering, for a sufficient duration, a therapeutically effective amount of DFMO, as measured by a reduction or stabilization of polyamine (spermine or spermidine) levels produced by the hyperplastic cells, together with a therapeutic effective amount of a second therapeutic agent selected from: an α-1 adrenergic receptor blocker such as terazosin, doxazosin, prazosin, indoramin, tamsulosin, prazicin and alfuzosin; a 5-α-reductase enzyme blocker such as finasteride; an azasteroid derivative; a combination of an α-1 adrenergic receptor blocker and a 5-α-reductase enzyme blocker; a potassium channel opener such as minoxidil; or a retinoic acid derivative. In a preferred embodiment, the second therapeutic agent is saw palmetto extract.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1 - Polyamine content in prostatic tissue obtained by core biopsy before (solid bar) and after (open bar) 28 days of DFMO. **FIG. 1A**. putrescine content; **FIG. 1B.** spermidine content; and **FIG. 1C.** spermine content.

FIG. 2 - Spermidine/spermine ratio in prostatic tissue obtained by core biopsy before (solid bar) and after (open bar) 28 days of DFMO.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

I. The Present Invention

This invention describes the use of DFMO (α-difluoromethylornithine) as a method for reducing the polyamine levels in the human prostate. DFMO is able to significantly decrease the levels of putrescine, spermidine (Spd) and spermine (Spm) in the prostate of male subjects given 28 days of oral DFMO. Thus, DFMO may be utilized as in therapeutic applications in the treatment of prostate cancer. Suprisingly, the amount and percent reduction of spermine levels in the prostate with the administration of DFMO are greater than in other tissues in patients receiving DFMO therapy, provided that the DFMO is administered in a sufficient amount and for a sufficient time duration to cause such a decrease.

II. DFMO (alpha-difluoromethylornithine)

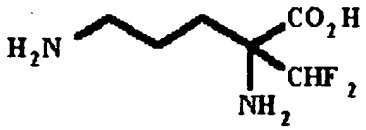
Numerous highly proliferative types of cancer are associated with increased levels of the polyamines putrescine, spermidine, and spermine in blood and urine. Studies have shown that this is related to increased polyamine synthesis by the rate-limiting enzyme, ornithine decarboxylase (ODC). Of particular interest in the instant application is prostate cancer.

The pathway for polyamine synthesis begins with L-ornithine. This natural amino acid, although not normally incorporated into proteins, is part of the urea cycle which metabolizes arginine to ornithine and urea. Ornithine is converted by ornithine decarboxylase (ODC) to putrescine and CO_2, the rate-limiting step in the production of polyamines. With the addition of propylamine donated from S-adenosylmethionine,

putrescine is converted to spermidine. Spermidine is then converted to spermine by spermine synthetase, again in association with the decarboxylation of S-adenosylmethionine. Putrescine, spermidine and spermine represent the three major polyamines in mammalian tissues. Polyamines are found in animal tissues and microorganisms and are known to play an important role in cell growth and proliferation. Although the exact mechanism of the role of the polyamines in cell growth and proliferation is not known, it appears that the polyamines may facilitate macromolecular processes such as DNA, RNA, or protein synthesis. Polyamine levels are known to be high in the testes, ventral prostate, and thymus, in psoriatic skin lesions, and in other cells undergoing rapid growth processes.

It also is well known that the rapid proliferation of tumor tissue is marked by an abnormal elevation of polyamine levels. Hence, the polyamines also may play an important role in the maintenance of tumor growth. Thus, ODC inhibitors, such as DFMO, may exert their therapeutic effect by blocking the formation of the polyamines and thereby slowing, interrupting, or arresting the proliferation and metastases of the tumor tissue.

DFMO (alpha-difluoromethylornithine, Eflornithine, Ornidyl®) is a structural analog of the amino acid L-ornithine and has a chemical structure: $C_6H_{12}N_2O_2F_2$



The DFMO can be employed in the methods of the invention as a racemic (50/50) mixture of D- and L-enantiomers, or preferably as a mixture of D- and L-isomers where the D-isomer is enriched relative to the L-isomer, for example, 70%, 80%, 90% or more by weight of the D-isomer relative to the L-isomer. Most preferably, the DFMO employed is substantially free of the L-enantiomer.

III. Anti-tumor Properties and Toxicity of DFMO

DFMO is relatively non-toxic to the host while producing inhibition of putrescine synthesis in tumors. Oral DFMO in humans is associated with ototoxicity (hearing loss) which limits recommended doses for chemoprevention to a single 0.5 gm/m^2 dose given daily. The other dose limiting toxic effect of DFMO is thrombocytopenia (abnormally few platelets in the blood), which occurs in about fifty percent of patients, leukopenia (abnormally few leukocytes), or anemia. Another side effect of DFMO is nausea and vomiting, which occurs in up to ninety percent of the patients. All these toxic effects are relatively harmless and reversible and cease upon withdrawal of the drug.

The effect of an ODC inhibitor for the control of the growth rate of rapidly proliferating tumor tissue has been assessed in standard animal tumor models. For example, the anti-tumor effect of DFMO has been demonstrated in the following animal tumor models: L1210 leukemia in mice, EMT6 tumor in Balb/C mice, 7,12-dimethylbenzanthracene-induced (DMBA-induced) mammary tumor in rats, and DFMO Morris 7288C or 5123 hepatoma in Buffalo rats. In addition, the anti-tumor effect of DFMO in combination with various cytotoxic agents has been demonstrated as follows: (a) in combination with vindesine or adriamycin in L1210 leukemia in mice, in Morris 7288C hepatoma in Buffalo rats, and in EMT6 tumor in mice, (b) in combination with cytosine arabinoside in L1210 leukemia in mice, (c) in combination with methotrexate in L1210 leukemia in mice, (d) in combination with cyclophosphamide in EMT6 tumor in mice and in DMBA-induced tumor in mice, (e) in combination with BCNU in mouse glioma 26 brain tumor, and (f) in combination with MGBG in L1210 leukemia in mice, in Morris 7288C hepatoma in Buffalo rats, in P388 lymphocytic leukemia in mice, and in S-180 sarcoma in mice.

Although DFMO can effectively block tumor putrescine biosynthesis, the resultant antitumor effect is cytostasis, not cytotoxicity. For example, DFMO reduces the growth rate of an MCA sarcoma, but does not produce tumor regression. This finding is consistent with reports of other investigators who showed that DFMO is a cytostatic agent. However, studies indicate that a significant role may exist for DFMO agents,

permitting the future development of combination chemotherapeutic regimens which incorporate DFMO.

The initial promise of DFMO as a therapeutic ODC inhibitor for use in the treatment of various neoplasias has dimmed somewhat because, although DFMO does, in fact, irreversibly inhibit ODC activity, cells treated *in vivo* with DFMO significantly increase their uptake of exogenous putrescine as described in U.S. Patent 4,925,835. The intercellular transport mechanisms of the cell do an "end run" around the DFMO-impaired ODC activity by importing putrescine from the extra-cellular milieu. Therefore, DFMO's effect *in vivo* is far poorer than *in vitro*. So, while DFMO treatment effectively inhibits intracellular putrescine neogenesis, it also results in increased uptake of extracellular putrescine, thereby offsetting its ODC inhibitory effect.

This problem is compounded by the fact that putrescine is present in many common foods, such as orange juice, which contains approximately 400 ppm putrescine. This makes it virtually impossible to provide a patient a nutritionally sufficient diet which is free of putrescine. Therefore, DFMO-treated cells are capable of importing sufficient amounts of extracellular putrescine to support cell division.

Strategies to make DFMO more acceptable to human patients are described in U.S. Patent 4,859,452 (incorporated by reference). Formulations of DFMO are described which include essential amino acids in combination with either arginine or ornithine to help reduce DFMO-induced toxicities.

Topical application of this drug to lesions that are superficial allow a chemopreventive cancer therapy that has minimal or no significant systemic uptake in human beings. One object of this invention is to provide a salve that can be used as a chronic topical chemopreventive agent against warts and superficial anogenital HPV lesions that are often precancerous. The salve also may be applied directly to cancerous lesions on the anogenital areas. It is known in the art that a topically applied, five percent solution of DFMO blocks the synthesis of DNA in mouse epidermis. *In vitro* putrescine levels decreased to twenty-five percent of control, while spermine and spermidine levels were not affected U.S. Patent 4,859,452 Further, application of a ten percent DFMO

cream in ten patients suffering from psoriasis was shown to reduce cutaneous spermine levels by sixty-six percent, with a marginal improvement in psoriatic lesions.

IV. D-DFMO

Toxicity of DFMO can be greatly reduced by using the D-enantiomer of DFMO, or mixtures of D- and L-isomers which are enriched for the D-isomer content such that the D-isomer comprises at least 60%, and preferably more than 90% by weight of the isomeric mixture. D-DFMO, while still an inhibitor of ODC, has lower toxicity, including ototoxicity, in animal models. In a study on guinea pigs, the enantiomers of DFMO did not show significant toxicity. The D-form of DFMO was found to have no significant effects on either the compound action potential or cochlear microphonic. An evaluation of auditory function found that the L- form of DFMO produced significant disruption of normal cochlear potentials.

The use of D-DFMO or enriched D-isomer mixtures can overcome many of the problems associated with the use of racemic (50/50) D,L-DFMO. D-DFMO or enriched D-DFMO isomer mixtures may be administered at a dosage higher than a racemic mixture, due to lower anticipated toxicity associated with the D enantiomer. In three separate studies using concentrations from 0.6 μM to 80 μM D-, L-, and D,L-DFMO, the effective concentration level of each which inhibits 50% of the ODC activity (K_i) was determined. Both enantiomers, as well as the racemic mixture, were inhibitory. The K_i of D-DFMO was four fold lower than the L-form and 3 fold lower than the mixture. (U.S. Patent Application entitled "D-enantiomer of DFMO and methods of use therefor," filed July 1, 2000).

V. DFMO and the Human Prostate

DFMO and its use in the treatment of benign prostatic hypertrophy are described in two patents, U.S. Patents 4,413,141 and 4,330,559. U.S. Patent 4,413,141 describes DFMO as being an inhibitor of ODC, both *in vitro* and *in vivo*. Administration of DFMO reportedly causes a decrease in putrescine and spermidine concentrations in cells in which these polyamines are normally actively produced. Additionally, DFMO has been shown to be capable of slowing neoplastic cell proliferation when tested in standard

tumor models. U.S. Patent 4,330,559 describes the use of DFMO and DFMO derivatives for the treatment of benign prostatic hypertrophy. Benign prostatic hypertrophy, like many disease states characterized by rapid cell proliferation, is accompanied by abnormal elevation of polyamine concentrations. The treatment described within this reference can be administered to a patient either orally, or parenterally. However, as pointed out above, a more recent study by Messing *et al.* (1999) failed to detect any reduction in spermidine or spermine levels in cancerous prostates after treatment with DFMO over a two-week period at 0.5 gm/m^2 given orally.

Recently the role of polyamines in prostate cancer has been revisited. Mohan *et al.* (1999) measured ODC activity in benign and malignant tissues from the same patient and found the cancerous portion to have levels almost three times that of benign tissue (1142 + 100 vs. 427 + 51). In addition they evaluated the ODC activity of prostatic fluid obtained by massage and found higher levels in men with prostate cancer compared to men with benign hypertrophy (3847 + 162 vs. 2742 +67).

Chemoprevention for prostate cancer is being studied using both hormonal and antioxidant agents. The first large scale chemopreventive study for prostate cancer began in 1993 by the Southwest Oncology Group (SWOG), and 18,000 men were randomized to finasteride (Proscar) or placebo for seven years. The hypothesis being tested was that a reduction in the levels of dihydrotestosterone (DHT) in the prostate by blocking the conversion of testosterone to DHT by 5 alpha reductatase would reduce prostate cancer. Results are expected to be available in 2004. A smaller study looked at surrogate end markers at time zero and after one year of Proscar treatment or no treatment (Cote *et al.*, 1998). The study group included men with an elevated PSA and negative prostate needle biopsies. The study demonstrated an increase in cancer diagnosed after a one year follow up biopsy in the men on Proscar (8 of 27 men vs. 1 of 25 men with no treatment (p=0.025)). A small Phase II study of oral fenretinide in 22 men at high risk for prostate cancer was undertaken by Pienta *et al.* (1997). This trial closed early when 8 men with negative prestudy biopsies developed malignancy while on study. With these two small studies unable to demonstrate protective benefits from the agent of interest, there should be a role in prostate cancer chemoprevention, for different agents, such as DFMO.

VI. Diagnosis of Prostate Cancer

The most commonly utilized current tests for prostate cancer are digital rectal examination (DRE) and analysis of serum prostate specific antigen (PSA). While PSA is specific to prostate tissue, it is produced by normal and benign as well as malignant prostatic epithelium, resulting in a high false-positive rate for prostate cancer detection (Partin & Oesterling, 1994).

Other markers that have been used for prostate cancer detection include prostatic acid phosphatase (PAP) and prostate secreted protein (PSP). PAP is secreted by prostate cells under hormonal control (Brawn *et al.*, 1996). It has less specificity and sensitivity than does PSA. As a result, it is used much less now, although PAP may still have some applications for monitoring metastatic patients that have failed primary treatments. In general, PSP is a more sensitive biomarker than PAP, but is not as sensitive as PSA (Huang *et al.*, 1993). Like PSA, PSP levels are frequently elevated in patients with BPH as well as those with prostate cancer.

Another serum marker associated with prostate disease is prostate specific membrane antigen (PSMA) (Horoszewicz *et al.*, 1987; Carter *et al.*, 1996; Murphy *et al.*, 1996). PSMA is a Type II cell membrane protein and has been identified as Folic Acid Hydrolase (FAH) (Carter *et al.*, 1996). Antibodies against PSMA react with both normal prostate tissue and prostate cancer tissue (Horoszewicz *et al.*, 1987). Murphy *et al.* (1995) used ELISA to detect serum PSMA in advanced prostate cancer. As a serum test, PSMA levels are a relatively poor indicator of prostate cancer. However, PSMA may have utility in certain circumstances. PSMA is expressed in metastatic prostate tumor capillary beds (Silver *et al.*, 1997) and is reported to be more abundant in the blood of metastatic cancer patients (Murphy *et al.*, 1996). PSMA messenger RNA (mRNA) is down-regulated 8-10 fold in the LNCaP prostate cancer cell line after exposure to 5-α-dihydroxytestosterone (DHT) (Israeli *et al.*, 1994).

As indicated earlier, DFMO acts late in the tumor promotion stage of epithelial carcinogenesis. A mechanism for the inhibition of colon carcinogenesis by DFMO includes suppression of the matrix metalloproteinase matrilysin (Wallon *et al.*, 1994), a

secreted protease which is involved in tumor invasion. This mechanism is operational in prostate cancer, since DFMO also suppresses interleukin-1β (IL-1β)-induced matrilysin expression in LNCaP human prostate cancer derived cells.

A relatively new potential biomarker for prostate cancer is human kallekrein 2 (HK2) (Piironen *et al.*, 1996). HK2 is a member of the kallekrein family that is secreted by the prostate gland. In theory, serum concentrations of HK2 may be of utility in prostate cancer detection or diagnosis, but the usefulness of this marker is still being evaluated.

VII. Methods of Treating Cancer

In a particular aspect, the present invention provides methods for the treatment of prostate cancer. Treatment methods will involve treating an individual with an effective amount of a therapeutic composition containing DFMO. An effective amount is described, generally, as that amount sufficient to detectably and repeatedly to ameliorate, reduce, minimize or limit the extent of a disease or its symptoms. More rigorous definitions may apply, including elimination, eradication or cure of disease.

DFMO may be administered at a dose of about 0.05 to about 20.0 gm/m^2/day. Preferred doses of DFMO to be administered are from about 0.1 to about 15.0 gm/m^2/day, or from about 0.1 to 12 gm/m^2/day, or from about 0.1 to 10 gm/m^2/day, or from about 0.1 to 8 gm/m^2/day, or from about 0.1 to 6 gm/m^2/day, or from about 0.1 to 4 gm/m^2/day, or from about 0.1 to 2 gm/m^2/day, or from about 0.1 to 1 gm/m^2/day, or from about 0.1 to 0.5 gm/m^2/day or about 0.5 gm/m^2/day. DFMO may also be administered at a dose of about 0.1 to 2.0 g/day, or from about 0.25 to 1.5 g/day, or more preferred from about 0.5 to 1.0 g/day.

To kill cells, inhibit cell growth, inhibit metastasis, decrease tumor size and otherwise reverse or reduce the malignant phenotype of tumor cells, using the methods and compositions of the present invention, one would generally contact a "target" cell with the therapeutic composition. This may be combined with compositions comprising other agents effective in the treatment of cancer. These compositions would be provided in a combined amount effective to kill or inhibit proliferation of the cell. This process

may involve contacting the cells with DFMO and the agent(s) or factor(s) at the same time. This may be achieved by contacting the cell with a single composition or pharmacological formulation that includes both agents, or by contacting the cell with two distinct compositions or formulations, at the same time, wherein one composition includes DFMO and the other includes the second agent.

Administration of the therapeutic DFMO composition of the present invention to a patient will follow general protocols for the administration of chemotherapeutics, taking into account the toxicity, if any, of DFMO. It is expected that the treatment cycles would be repeated as necessary. It also is contemplated that various standard therapies, as well as surgical intervention, may be applied in combination with the described therapy.

Where clinical application of a DFMO therapy is contemplated, it will be necessary to prepare the complex as a pharmaceutical composition appropriate for the intended application. Generally this will entail preparing a pharmaceutical composition that is essentially free of pyrogens, as well as any other impurities that could be harmful to humans or animals. One also will generally desire to employ appropriate salts and buffers to render the complex stable and allow for complex uptake by target cells.

Aqueous compositions of the present invention comprise an effective amount of the compound, dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. Such compositions can also be referred to as inocula. The phrases "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, or a human, as appropriate. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

The compositions of the present invention may include classic pharmaceutical preparations. Dispersions also can be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

Depending on the particular cancer to be, administration of therapeutic compositions according to the present invention will be *via* any common route so long as the target tissue is available *via* that route. This includes oral, nasal, buccal, rectal, vaginal or topical. Topical administration would be particularly advantageous for treatment of skin cancers. Alternatively, administration will be by orthotopic, intradermal, subcutaneous, intramuscular, intraperitoneal or intravenous injection. Such compositions would normally be administered as pharmaceutically acceptable compositions that include physiologically acceptable carriers, buffers or other excipients.

In certain embodiments, *ex vivo* therapies also are contemplated. *Ex vivo* therapies involve the removal, from a patient, of target cells. The cells are treated outside the patient's body and then returned.

The treatments may include various "unit doses." Unit dose is defined as containing a predetermined-quantity of the therapeutic composition calculated to produce the desired responses in association with its administration, *i.e.*, the appropriate route and treatment regimen. The quantity to be administered, and the particular route and formulation, are within the skill of those in the clinical arts. Also of import is the subject to be treated, in particular, the state of the subject and the protection desired. A unit dose need not be administered as a single injection but may comprise continuous infusion over a set period of time.

One of the preferred embodiments of the present invention involves the use of therapeutic compositions of DFMO with specific target cancer cells. Of particular interest are prostate cancer cells.

According to the present invention, one may treat the cancer by directly injection a tumor with the DFMO or analog composition. Alternatively, the tumor may be infused

or perfused with the composition using any suitable delivery vehicle. Local or regional administration, with respect to the tumor, also is contemplated. Finally, systemic administration may be performed. Continuous administration also may be applied where appropriate, for example, where a tumor is excised and the tumor bed is treated to eliminate residual, microscopic disease. Delivery *via* syringe or catherization is preferred. Such continuous perfusion may take place for a period from about 1-2 hours, to about 2-6 hours, to about 6-12 hours, to about 12-24 hours, to about 1-2 days, to about 1-2 wk or longer following the initiation of treatment. Generally, the dose of the therapeutic composition *via* continuous perfusion will be equivalent to that given by a single or multiple injections, adjusted over a period of time during which the perfusion occurs. For tumors of > 4 cm, the volume to be administered will be about 4-10 ml (preferably 10 ml), while for tumors of < 4 cm, a volume of about 1-3 ml will be used (preferably 3 ml). Multiple injections delivered as single dose comprise about 0.1 to about 0.5 ml volumes. The viral particles may advantageously be contacted by administering multiple injections to the tumor, spaced at approximately 1 cm intervals.

In certain embodiments, the tumor being treated may not, at least initially, be resectable. Treatments with therapeutic DFMO compositions may increase the resectability of the tumor due to shrinkage at the margins or by elimination of certain particularly invasive portions. Following treatments, resection may be possible. Additional treatments subsequent to resection will serve to eliminate microscopic residual disease at the tumor site.

Other factors that cause DNA damage and have been used extensively include what are commonly known as γ-rays, X-rays, and/or the directed delivery of radioisotopes to tumor cells. Other forms of DNA damaging factors are also contemplated such as microwaves and UV-irradiation. It is most likely that all of these factors effect a broad range of damage on DNA, on the precursors of DNA, on the replication and repair of DNA, and on the assembly and maintenance of chromosomes. Dosage ranges for X-rays range from daily doses of 50 to 200 roentgens for prolonged periods of time (3 to 4 wk), to single doses of 2000 to 6000 roentgens. Dosage ranges for

radioisotopes vary widely, and depend on the half-life of the isotope, the strength and type of radiation emitted, and the uptake by the neoplastic cells.

VIII. Combinational Therapy with DFMO

The present invention contemplates that DFMO may be used in combination with other therapies. There is an increasing body of experimental and epidemiological data suggesting that aspirin, and some other NSAIDs, exert a chemopreventive action on colorectal cancers and maybe also on stomach, esophagus (Thun *et al.*, 1993) and even bladder (Earnest *et al.*, 1992) cancers. Aspirin, ibuprofen, piroxicam (Reddy *et al.*, 1990; Singh *et al.*, 1994), indomethacin (Narisawa, 1981), and sulindac (Piazza *et al.*, 1997; Rao *et al.*, 1995), effectively inhibit colon carcinogenesis in the azoxymethane AOM-treated rat model and flurbiprofen has demonstrated anti-tumor effects in the APC(Min)+ mouse model (Wechter *et al.*, 1997). NSAIDs also inhibit the development of tumors harboring an activated Ki-*ras* (Singh and Reddy, 1995). Studies have been conducted in which DFMO was combined with aspirin to evaluate its chemopreventive effect in to AOM-treated rats. The combination of aspirin and DFMO administered after AOM was found to be synergistic (Li *et al.*, 1999). The results demonstrated that the aspirin and DFMO combination could prevent colon cancer when administered after AOM (Li *et al.*, 1999).

The combination of DFMO with the chemotherapeutic agent piroxicam has been shown to have a synergistic chemopreventive effect in the AOM-treated rat model of colon carcinogenesis (Reddy *et al.*, 1990), although DFMO exerted a greater suppressive effect than piroxicam on Ki-*ras* mutation and tumorigenesis when each agent was administered separately (Singh *et al.*, 1993; Reddy *et al.*, 1990; Kulkarni *et al.*, 1992). In one study, administration of DFMO or piroxicam to AOM-treated rats reduced the number of tumors harboring Ki-*ras* mutations from 90% to 36% and 25% respectively (Singh *et al.*, 1994). The Apc mutant Min mouse model was used to test piroxicam and DFMO to determine that combined treatment was much more effective than either agent alone and resulted in a significant number of mice totally free of any intestinal adenomas (Jacoby *et al.*, 2000). Both DFMO and piroxicam also reduced the amount of biochemically active p21 *ras* in existing tumors. (Singh *et al.*, 1993). Despite the

success of the drugs in model systems, phase I trials conducted with this combination resulted in a range of adverse side effects (Carbone *et al.*, 1998).

Because DFMO is an effective inhibitor of ODC, some researchers are attempting to use DFMO as part of a conjunctive treatment in combination with interferon. U.S. Patent 4,499,072, describe improving the polyamine-depletion effects of ODC inhibitors (including DFMO) by using interferon in combination with the ODC inhibitor. Additionally, it describes the use of both an ODC inhibitor and interferon in conjunction with a known cytotoxic agent such as methotrexate. U.S. Patent 5,002,879, describe a similar conjunctive therapy in which an ODC inhibitor, preferably DFMO, is used in combination with lymphokine-activated killer (LAK) cells and interleukin-2.

Cancer therapies also include a variety of combination therapies of DFMO with both chemical and radiation based treatments. Combination chemotherapies include, for example, cisplatin (CDDP), carboplatin, procarbazine, mechlorethamine, cyclophosphamide, camptothecin, ifosfamide, melphalan, chlorambucil, bisulfan, nitrosurea, dactinomycin, daunorubicin, doxorubicin, bleomycin, plicomycin, mitomycin, etoposide (VP16), tamoxifen, taxol, transplatinum, 5-fluorouracil, vincristin, vinblastin and methotrexate or any analog or derivative variant thereof. For precancerous conditions such as benign prostatic hyperplasia, a second therapeutic agent selected from α-1 adrenergic receptor blocker such as terazosin, doxazosin, prazosin, bunazosin, indoramin, tamsulosin, prazicin or alfuzosin; a 5-α-reductase enzyme blocker such as finasteride or an azasteroid derivative; a combination of an α-1 adrenergic receptor blocker, and a 5-α-reductase enzyme blocker, a potassium channel opener such as minoxidil, and a retinoic acid derivative. In a preferred embodiment, the second therapeutic agent is saw palmetto extract.

Various combinations may be employed, for instance where DFMO composition is "A" and the radio-, chemotherapeutic or other therapeutic agent is "B":

A/B/A B/A/B B/B/A A/A/B A/B/B B/A/A A/B/B/B B/A/B/B

B/B/B/A B/B/A/B A/A/B/B A/B/A/B A/B/B/A B/B/A/A

B/A/B/A B/A/A/B A/A/A/B B/A/A/A A/B/A/A A/A/B/A

The terms "contacted" and "exposed," when applied to a cell, are used herein to describe the process by which a therapeutic composition and a chemotherapeutic or radiotherapeutic agent are delivered to a target cell or are placed in direct juxtaposition with the target cell. To achieve cell killing or stasis, both agents are delivered to a cell in a combined amount effective to kill the cell or prevent it from dividing.

The DFMO therapy may precede or follow the other agent treatment by intervals ranging from minutes to weeks. In embodiments where the other agent and DFMO are applied separately to the cell, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the agent and DFMO would still be able to exert an advantageously combined effect on the cell. In such instances, it is contemplated that one would contact the cell with both modalities within about 12-24 h of each other and, more preferably, within about 6-12 h of each other, with a delay time of only about 12 h being most preferred. In some situations, it may be desirable to extend the time period for treatment significantly, however, where several days (2, 3, 4, 5, 6 or 7) to several weeks (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations.

IX. Pharmaceutical Compositions

Aqueous compositions of the present invention comprise an effective amount of DFMO, dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. The phrases "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, or a human, as appropriate.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions. For human administration, preparations should meet

sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

The biological material should be extensively dialyzed to remove undesired small molecular weight molecules and/or lyophilized for more ready formulation into a desired vehicle, where appropriate. The active compounds will then generally be formulated for parenteral administration, *e.g.*, formulated for injection via the intravenous, intramuscular, sub-cutaneous, intralesional, or even intraperitoneal routes. The preparation of an aqueous composition that contains an headpin agent as an active component or ingredient will be known to those of skill in the art in light of the present disclosure. Typically, such compositions can be prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for using to prepare solutions or suspensions upon the addition of a liquid prior to injection can also be prepared; and the preparations can also be emulsified.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

DFMO of the present invention can be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts, include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids

such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. The preparation of more, or highly, concentrated solutions for direct injection is also contemplated, where the use of DMSO as solvent is envisioned to result in extremely rapid penetration, delivering high concentrations of the active agents to a small area.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, but drug release capsules and the like can also be employed.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

In addition to the compounds formulated for parenteral administration, such as intravenous or intramuscular injection, other pharmaceutically acceptable forms include, *e.g.*, tablets or other solids for oral administration; liposomal formulations; time release capsules; and any other form currently used, including cremes.

One also may use nasal solutions or sprays, aerosols or inhalants in the present invention. Nasal solutions are usually aqueous solutions designed to be administered to the nasal passages in drops or sprays. Nasal solutions are prepared so that they are similar in many respects to nasal secretions, so that normal ciliary action is maintained. Thus, the aqueous nasal solutions usually are isotonic and slightly buffered to maintain a pH of 5.5 to 6.5. In addition, antimicrobial preservatives, similar to those used in ophthalmic preparations, and appropriate drug stabilizers, if required, may be included in the formulation. Various commercial nasal preparations are known and include, for example, antibiotics and antihistamines and are used for asthma prophylaxis.

Additional formulations which are suitable for other modes of administration include vaginal suppositories and pessaries. A rectal pessary or suppository may also be used. Suppositories are solid dosage forms of various weights and shapes, usually medicated, for insertion into the rectum, vagina or the urethra. After insertion, suppositories soften, melt or dissolve in the cavity fluids. In general, for suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1%-2%.

Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders. In certain defined embodiments, oral pharmaceutical compositions will comprise an inert diluent or assimilable edible carrier, or they may be enclosed in hard or soft shell gelatin capsule, or they may be compressed into tablets, or they may be incorporated directly with the food of the diet. For oral therapeutic administration, the active compounds may be incorporated with excipients and used in the form of ingestible tablets, buccal tables, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 0.1% of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 2 to about 75% of the weight of the unit, or preferably between 25-60%. The amount of active compounds in such therapeutically useful compositions is such that a suitable dosage will be obtained.

The tablets, troches, pills, capsules and the like may also contain the following: a binder, as gum tragacanth, acacia, cornstarch, or gelatin; excipients, such as dicalcium phosphate; a disintegrating agent, such as corn starch, potato starch, alginic acid and the like; a lubricant, such as magnesium stearate; and a sweetening agent, such as sucrose, lactose or saccharin may be added or a flavoring agent, such as peppermint, oil of wintergreen, or cherry flavoring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may

be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup of elixir may contain the active compounds sucrose as a sweetening agent methyl and propylparabens as preservatives, a dye and flavoring, such as cherry or orange flavor.

In certain embodiments, the use of liposomes and/or nanoparticles is contemplated for the formulation and administration of DFMO or an analog thereof. The formation and use of liposomes is generally known to those of skill in the art, and is also described below.

Nanocapsules can generally entrap compounds in a stable and reproducible way. To avoid side effects due to intracellular polymeric overloading, such ultrafine particles (sized around 0.1 μm) should be designed using polymers able to be degraded *in vivo*. Biodegradable polyalkyl-cyanoacrylate nanoparticles that meet these requirements are contemplated for use in the present invention, and such particles may be are easily made.

Liposomes are formed from phospholipids that are dispersed in an aqueous medium and spontaneously form multilamellar concentric bilayer vesicles (also termed multilamellar vesicles (MLVs). MLVs generally have diameters of from 25 nm to 4 μm. Sonication of MLVs results in the formation of small unilamellar vesicles (SUVs) with diameters in the range of 200 to 500 Å, containing an aqueous solution in the core.

The following information may also be utilized in generating liposomal formulations. Phospholipids can form a variety of structures other than liposomes when dispersed in water, depending on the molar ratio of lipid to water. At low ratios the liposome is the preferred structure. The physical characteristics of liposomes depend on pH, ionic strength and the presence of divalent cations. Liposomes can show low permeability to ionic and polar substances, but at elevated temperatures undergo a phase transition which markedly alters their permeability. The phase transition involves a change from a closely packed, ordered structure, known as the gel state, to a loosely packed, less-ordered structure, known as the fluid state. This occurs at a characteristic phase-transition temperature and results in an increase in permeability to ions, sugars and drugs.

Liposomes interact with cells via four different mechanisms: Endocytosis by phagocytic cells of the reticuloendothelial system such as macrophages and neutrophils; adsorption to the cell surface, either by nonspecific weak hydrophobic or electrostatic forces, or by specific interactions with cell-surface components; fusion with the plasma cell membrane by insertion of the lipid bilayer of the liposome into the plasma membrane, with simultaneous release of liposomal contents into the cytoplasm; and by transfer of liposomal lipids to cellular or subcellular membranes, or *vice versa*, without any association of the liposome contents. Varying the liposome formulation can alter which mechanism is operative, although more than one may operate at the same time.

X. Examples

The following example is included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Example 1 - Experimental

This protocol was approved by the investigational review board of the University of California Irvine and the Long Beach Veterans Administration Medical Center. Men between the ages of 50 and 85, who were undergoing a trans-rectal prostate needle biopsy for either an elevated PSA or abnormal rectal exam, signed a written consent to undergo four additional core needle biopsies of the peripheral zone at the time of their routine extant biopsy. The additional four biopsies were immediately frozen liquid nitrogen and stored in the minus 70-degree freezer. The sextent biopsy was sent to Pathology for routine analysis. If the patient elected (1) radical surgery for prostate cancer (2) transurethral resection for outlet obstruction, or (3) a second biopsy due to the diagnosis of atypia, he was then asked to continue participation in the trial and to take oral DFMO

0.5gm/m^2 for 28 days prior to and the day of the second procedure. Patients were monitored for side effects using questionnaires and interview techniques. Coagulation parameters were carefully assessed prior to surgery. In the operating room just prior to the surgical procedure four trans-rectal core biopsies of the peripheral zone were taken and frozen. The pre and post DFMO specimens were sent together to Arizona on dry ice for histology and polyamine analysis. Radical prostatectomy specimens were staged according to the TMN updated 1997 staging system (American Joint Committee on Cancer 1997).

To determine prostate core polyamine contents, cores were first thawed, washed in phosphate buffered saline (PBS) and weighted. Samples were then minced in 0.2 N perchloric acid and sonicated to disrupt cellular material. Samples were centrifuged at 2,000 X g for 5 minutes to separate acid soluble and insoluble fractions. The acid soluble fraction was evaluated for polyamine contents, using reverse-phase, ion-pared high performance liquid chromatography (PHLC) methods, as described elsewhere (Gerner *et al.* 1994; Meyskens *et al* 1994; Meyskens *et al.* 1998). Briefly, polyamines are separated on a C18 B Ondapak column, derivatized with o-pthaldehyde after separation and detected by absorption of the derivatized material at 750 nm.

Mean, median, and range for polyamine levels are reported in nmol/mg protein. The limit of detection of our method is 0.01 nmol/mg.

Routine sextant biopsies and surgical specimens were processed and reported according to the protocol established by the department of Pathology at the Long Beach Veterans Administration Medical Center.

Histological Changes were assessed by bright field microscopy methods. Pre- and post DFMO polyamine values were compared using the Wilcoxon matched-pairs signed rank test. Thus, for each patient we were able to take into account both the magnitude and the direction of changes in polyamines due to DFMO.

Example 2 - Results

Demographics: Forty-nine men signed consents for participation in the study. Twenty-two had the first set of four additional cores at time of sextant biopsy. Based on the routine biopsy results and patient consent ten men were started on DFMO. Nine men had the second set of biopsies. One man elected to receive no treatment for his prostate cancer after starting the DFMO. The detailed demographics of the participants and their routine biopsy histopathology results are presented in Table 1.

The mean age of men who completed the trial was 65.6 years (median 66 years range 56-73 years). Ethnicity of the group was White (3), Latino (3), Black (1), Asian (1), and mixed White/Asian (1). Indications for the biopsy were elevated PSA (5), and abnormal digital rectal exam (3) or both (1). The average PSA before the first biopsy for the nine patients was 9.4 ng/ml, (median 5.1 ng/ml range 1.7-47.2) DFMO was given an average of 28.2 days (median 28 days, range 21-35 days). Compliance was 100% as measured by documentation and interview. Second procedures were radical retropubic prostatectomy (RRR) (4), repeat biopsy (4), and transurethral resection of the prostate (TURP) (1).

Side Effects: Side effects were reported in four men. Two men had grade 0 side effects involving mild clinical hearing loss (not confirmed by pure tone audiology), nausea, diarrhea, and fatigue in one man, and a sudden one-time weakness in another man. One man had grade 1 vertigo, along with nausea and epigastric pain. There was no clinically significant change in the platelet counts, or protime with the DFMO. Bleeding time was checked prior to surgery and was not altered by DFMO.

Pathology: (Table 1) Routine pathological diagnosis on the first biopsy was atypia (2), atypia with inflammation (3), Gleason sum 5 (1), Gleason sum 6 (2), and Gleason sum 7 (1) (total 9). The routine pathology specimens at the second procedure demonstrated BPH (1). BPH and inflammation (1), inflammation (1), atypia (1), Gleason sum 6 (1), Pt2A Gleason sum 6 (1), pT2b Gleason sum 6 (1), pT3a Gleason sum 6 (1), and pT4 Gleason sum 8 (1), (total 9).

TABLE 1

Patient Demographics

Patient	1	2	3	4	5	6	7	8	9
Age	56	66	73	69	70	62	67	63	65
Race	Black	White	Latino	Asian/White	White	White	Asian	Latino	Latino
DRE	Normal	Normal	Normal	Abnormal	Abnormal	Normal	Normal	Normal	Normal
PSA	5.8	9.8	2.3	1.7	5.1	2.6	6.3	47.2**	4.1
Biopsy Pathology	Atypia	Gleason 5	Atypia	Atypia	Atypia	Gleason 6	Atypia	Gleason 7	Gleason 6
			Inflammation		Inflammation		Inflammation		
Study PSA	4.4	Not done	2.3	1.5	16.6*	11.1*	8.7	23.2	6.8*
Days of DFMO	28	28	35	30	30	28	25	29	21
Pre-op PSA	3.7	7.5	2.5	1.4	5	2.3	6.8	35.5	8.2*
Procedure	Biopsy	RRP	Biopsy	Biopsy	TURP	RRP	Biopsy	RRP	RRP
Pathology	Atypia	T3a Gleason 6	Gleason 6	BPH	BPH Inflammation	T2b Gleason 6	Inflammation	T4 Gleaosn 8	T2a Gleason 6

Core Histology: No major differences in histology were observed in core biopsy samples obtained before or after DFMO treatment. (RB Nagle, personal communication, data not shown).

PSA: (Table 1) The average PSA before the first biopsy for the nine patients was 9.4 ng/ml, (median 5.1 ng/ml range 1.7-47.2) For the four patients with a final benign pathology, we compared the pre-biopsy PSA or pre-DFMO PSA with the PSA drawn while on DFMO. The PSA decreased in each patient (patients 1,4,5,7). The PSA increased in 3 of 5 of the patients with malignant pathology (patients 3,8,9). Although of these three men, one man's PSA was very variable (patient 8), and another man's PSA was drawn within 6 weeks of the biopsy and thus the PSA may have been falsely elevated from the biopsy (patient 9).

Polyamine Levels: FIG. 1 compares the values of putrescine, spermidine, spermine, and the spermidine/spermine ratio in the nine men who had biopsies performed before and after oral DFMO. Pre-therapy putrescine was detectable prior to DFMO in six men and non-detectable (<0.01 nmol/mg) in three men. The average putrescine level was 0.42 nmol/mg (median 0.27, range nd to 0.94). All men had undetectable levels of putrescine after DFMO. For the six men with pre-DFMO putrescine level ≥0.01 the average decrease from baseline was 97.6%, (p=0.031). Spermidine was measurable in all specimens prior to DFMO. The average pretreatment level was 1.21 nmol/mg (median 0.81, range 0.49 to 3.82), and decreased in all specimens after DFMO. The average level of spermidine after therapy was 0.32 (median 0.21, range nd to 1.18), and in two specimens the levels were undetectable. The average percent decrease from baseline was 73.6% (p=0.004). The spermine levels prior to DFMO were the highest of all the polyamines tested. Average spermine level prior to DFMO was 29.14 nmol/mg (median 28.85 range 9.88 to 53.66) and after DFMO decreased in all specimens to an average level of spermine, 14.33 (median 17.40, range 3.24 to 25.58). The average decrease from baseline was 50.8% (p=0.004). The spermidine/spermine ratio was calculated for each specimen. Eight of nine patients had a decrease in this ratio after DFMO was given. The average decrease from baseline was 50% (median 52% range – 25.3% to 97%) (p=0.019). The two patients with extracapsular prostate cancer had the least decrease from baseline

or an actual increase in spermidine/spermine ratio after treatment with DFMO (patients 2 and 8).

Example 3 - Discussion

In this short-term trial, the inventors were able to demonstrate a significant reduction in the prostate polyamines: putrescine, spermidine and spermine and the spermidine/spermine ratio after administration of oral DFMO 0.5gm/m^2 daily for 28 days. Putrescine decreased from baseline by 98% (n=6, p=0.031), spermidine by 74% (n=9, p=0.004), and spermine by 51% (n=9, p=0.004). Particularly intriguing was the demonstration of a large reduction in spermine. This effect of DFMO on spermine levels has not been observed in other tissues in patients receiving DFMO on spermine levels has not been observed in other tissues in patients receiving DFMO therapy (Gerner *et al.*, 1994; Meyskens *et al.*, 1994; Meyskens *et al.*,1998).

A brief review of the inventor's trial design and that of Messing *et al.* (1999), discussed *supra*, points to study differences that can account for the discordant results between the two studies. An advantage of the current study was that the inventors elected to use each male as his own control for polyamine suppression by using samples from the same male before and after DFMO. In addition, the samples were not run until both the before and after samples could be run together to avoid batch differences. The data shows a wide variation in polyamine levels among the subjects prior to manipulation, with putrescine demonstrating the least variability of the polyamines tested. (Put (range non-detectable (n.d.<0.01)-0.94), Spd (range 0.49-3.82), and Spm (range 9.96-53.7)). This variability makes it difficult to assess differences in a small control vs. treatment group, and may be the answer as to why only putrescine, with the smallest variability, was significantly changed in the Messing trial. In the inventors' trial, each man was his own control, and thus the effects of DFMO were more easily measured. Similar difficulty with the variability in polyamine levels was addressed by Mitchell *et al.* (1997). They reported on polyamine levels in cervical cancer compared to normal cervical tissue, specifically addressing the potential of using polyamine levels as intermediate markers. Although there were differences in the polyamine levels between these types of tissues,

the authors concluded that due to the variability of the polyamine levels large numbers of subject would be needed to see a significant result.

There are also processing issues related to the manner in which the tissues were managed between these two studies. The inventors specified peripheral biopsies of the prostate, as the ODC activities are different in the different prostate lobes of the rat (they are higher in the ventral lobe as compared to the dorsal lobe) and it was not known if these same differences would be observed in the zones of the human prostate (Heston *et al.*, 1982). In addition, the biopsies taken after 28 days of DFMO just prior to surgery were carried out just as they had been initially, transrectally and frozen, to avoid any potential polyamine degradation or alteration that may occur with cautery or devasularization of the prostate during surgery. It is unknown what the impact of ischemia for one to two hours on the prostate is as it is sytematically devasularized and removed. The unknown effect of this manipulation is obviated in the study by taking the cores prior to definitive surgery.

Another difference between the inventor's study and that of Messing *et al.* (1999) is the length of treatment. The subjects received 4 wks of DFMO (vs 2 wks). Work in the rat prostate published by Danzin demonstrated that the most significant ODC inhibition to 10% of the control value by intraperitoneal injection of DFMO (100 mg/kg every 12 hrs) was seen after 4 to 6 hrs. Inhibition rose to 50% reduction compared to control by 24 hrs. Through out the rest of the 8-day study inhibition ranged from 30 to 50% from the control and did not reach a steady state. In the same paper Danzin measured polyamine levels after two weeks of treatment and found significant reduction of putrescine, and spermine levels at low dos DFMO (100 mg/kg) and spermidine levels at the higher dose (1 gm/kg) (Danzin *et al.*, 1979). The reduction in putrescine from 202 $\pm$ 37 to 4 $\pm$ 4 nmol/g after two weeks of 100 mg/kg DFMO makes it difficult to speculate on any further impact of another 2 wks of treatment on putrescine. But there was less of a reduction in spermidine (7334 $\pm$ 193 to 1188 $\pm$ 289) and no significant reduction in spermine (2968 $\pm$ 293 to 2741 $\pm$ 346) at two weeks using 100 mg/kg DFMO. It may be that these later two polyamines require longer treatment times to see a reduction with DFMO (Danzin *et al.* 1979).

The polyamine levels were evaluated with respect to race, age, pathology, and days of inhibition with no obvious trends noted in this small series. The apparent lack of reduction in the spermidine/spermine ratios with DFMO in the men with extra-prostatic cancer is of great interest. Of the five men with prostate cancer, two men were found to have prostate cancer outside the prostate gland (extracapsular and bladder neck invasion T3 and T4) and these two men (patients 2 and 8) had little or an actual increase in the spermidine/spermine ratio with DFMO. Whereas the specimens with prostate cancer confined to the gland (T2, organ confined) had a similar reduction in the spermidine/spermine ratio as that seen with the benign prostate disease (FIG. 2). It is possible that as the tumor becomes more aggressive, as seen with extracapsular and bladder neck invasion, alternative polyamine synthesis or the dysregulation of polyamines occurs. Follow-up of this observation in a larger cohort would be of great interest. The polyamine dysregulation could be predictive of a poor prognosis and that the measured dysregulation of polyamines could be used to determine which patients need to be treated in a more aggressive manner or just be observed. A further study would help determine whether the invasion into other tissues or the dysregulation of polyamines occurs first. It is also believed that there is a time before extraprostatic cancer extension when there is measured dysregulation of polyamines.

REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

Auvinen, Paasinen, Andersson, Holtta, "Ornithine decarboxylase activity is critical for cell transformation," *Nature*, 360:355-358, 1992.

Auvinen, M., "Cell transformation, invasion and angiogenesis: a regulatory role for ornithine decarboxylase and polyamines?" *J. Natl Cancer Inst.*, 89:533-537, 1997.

Brawn *et al.*, *The Prostate*, 28: 295-299, 1996.

Carbone, Douglas, Larson, Verma, Blair, Pomplun, Tutsch, "Phase I chemoprevention study of piroxicam and alpha- difluoromethylornithine." *Cancer Epidemiol Biomarkers Prev* 7(10): 907-12, 1998.

Carter *et al.*, *Proc. Nat'l Acad. Sci. USA* 93: 749-753, 1996.

Clifford, Morgan, Yupsa, Soler, Gilmour, "Role of ornithine decarboxylase in epidermal tumorigenesis," *Cancer Res.*, 55:4205-4209, 1995.

Cote, Skinner, Salem, Mertex, Stanczyke, Henderson, Pike, Ross, "The effect of finasteride on the prostate gland in men with elevated serum prostate specific antigen levels," *Br. J. Cancer*, 78(3):413-418, 1998.

Croghan, Aicken, Meyskens, "Dose-related α-difluoromethylornithine ototoxicity," *Am. J. Clin. Oncol.*, (14):331-5, 1991.

Danzin, Jung, Grove, Bey, "Effect of a-difluoromethylornithne, an enzyme-activated irreversible inhibitor of ornithine decarboxylase, on polyamine levels in rat tissues," *Life Sci.*, 24:519-524, 1979.

Danzin, Jung, Claverie, Grove, Sjoerdsma, Koch-Weser, "Effects of α-difluoromethylornithine, an enzyme-activated irreversible inhibitor of ornithine decarboxylase, on testosterone-induced regeneration of prostate and seminal vesicle in castrated rats," *Biochem. J.*, 180:507-513, 1979.

Dunzendorfer and Russell, "Altered polyamine profiles in prostatic hyperplasia and in kidney tumors," *Cancer Res.*, 38:2321-2324, 1978.

Feuer, Merrill, Hankey, "Cancer surveillance series: interpreting trends in prostate cancer-part II: Cause of death misclassification and the recent rise and fall in prostate cancer mortality," *J. Natl Cancer Inst.*, 91(12):1025-1032, 1999.

Garewal, Sampliner, Gerner, Steinbronn, Alberts, Kendall, "Ornithine decarboxylase activity in Barrett's esophagus: a potential marker for dysplasia," *Gastroneterology*, 94:819-821, 1988.

Gerner, Garewal, Emerson, Sampliner, "Gastrointestinal tissue polyamine contents of patients with Barretts esophagus treated with difluoromethylornithine," *Cancer Epidemiol. Biomarkers Prev.*, 3:325-330, 1994.

Heston, Kadmon, Lazan, Fair, "Copenhagen rat prostatic tumor ornithine decarboxylase activity (ODC) and the effect of the ODC inhibitor α-difluoromethylornithine," *The Prostate*, 3:383-389, 1982.

Horoszewicz, Kawinski and Murphy, *Anticancer Res.*, 7:927-936, 1987.

Huang *et al.*, *Prostate*, 23: 201-212, 1993.

Israeli *et al.*, *Cancer Research*, 54:1807-1811, 1994.

Jacoby *et al.*, "Chemopreventive efficacy of combined piroxicam and difluoromethyl-ornithine treatment of Apc mutant Min mouse adenomas, and selective toxicity against Apc mutant embryos," *Cancer Research*, 60(7): 1864-70, 2000.

Kergozien, "Polyamine deprivation," *Life Sci.*, 58(24):2209-2215, 1996.

Kubota, Kiyosawa, Nomura, Yamada, Seyama, "Ornithine decarboxylase overexpression in mouse 10T1/2 fibroblasts: cellular transformation and invasion," *J. Nat'l Cancer Inst.*,(89):567-71, 1997.

Kulkarni, Zang, Kelloff, Reddy, "Effect of the chemopreventive agents piroxicam and D,L-α-difluoromethylornithine on intermediate biomarkers of colon carcinogenesis," *Carcinogenesis*, (13):995-1000, 1992.

Levin, Chamberlain, Prados, Choucair, Berger, Silver, *et al.*, "Phase I-II study of eflornithine and mitogauzone combined in the treatment of recurrent primary brain tumors," *Cancer Treat. Rep.*, (71):459-64, 1987.

Li , Schut, Conran , Kramer, Lubet, Steele, Hawk, Kelloff, Pereira, "Prevention by aspirin and its combination with alpha- difluoromethylornithine of azoxymethane-

induced tumors, aberrant crypt foci and prostaglandin E2 levels in rat colon." *Carcinogenesis* 20(3): 425-30, 1999.

Luk and Baylin, "Ornithine decarboxylase as a biologic marker in familial colonic polyposis," *N. Eng. J. Med.*, 311:980-983, 1984.

McCann, Pegg, "Ornithine decarboxylase as an enzyme target for therapy," *Pharmacol. Ther.*, 54:195-215, 1992.

Messing, Love, Tutsch, Verma, Douglas, Pomplun, Simsiman, Wilding, "Low dose difluoromethylornithine and polyamine levels in human prostate tissue," *J. Natl Cancer Inst.*, 16:1416-1417, 1999.

Meyskens, Emerson, Pelot, Meshkinpour, Shassetz, Einsahr, Alberts, Gerner, "Dose de-escalation chemoprevention trial α-difluoromethlornithine in patients with colon polyps," *J. Natl Cancer Inst.*, 86(15):1122-1130, 1994.

Meyskens, Gerner, Emerson, Pelot, Durbin, Doyle, Lagerberg, "Effect of difluoromethylornithine on rectal mucosal levels of polyamines in a randomized, double-blind trial for colon cancer prevention" *J. Natl. Cancer Inst.*, 90(16):1212-8, 1998.

Meyskens, Kingsley, Glatrke, Loescher, Booth, "A phase II study of α-difluoromethylornithine (DFMO) for the treatment of metastatic melanoma," *Invest. New Drugs*, (4):257-62, 1986.

Meyskens and Gerner, "Development of difluoromethylornithine (DFMO) as a chemopreventive agent," *Clin. Cancer Res.*, 5:945-951, 1999.

Mitchell, Tortolero-Luna, Lee, Hittelman, Lotan, Wharton, Hong, Nishioka, "Polyamine measurements in the uterine cervix," *J. Cell Biochem Suppl*, 28-29:125-132, 1997.

Mohan, Challa, Gupta, Bostwick, Ahmad, Agarwal, Marengo, Amini, Paras, MacLennan, Resnick, Mukhtar, "Over-expression of ornithine decarboxylase in prostate cancer and prostatic fluid in humans," *Clin. Caner Res.*, 5:143-147, 1999.

Moshier, Dosescu, Skunca, Luc, "Transformation of NIH/3T3 cells by ornithine decarboxylase over-expression," *Cancer Res.*, 53:2618-2622, 1993.

Moulinoux, Wuemener, Cipolla, Guille, Havouis, Martin, Lobel, Seiler, "The growth of MAT-LyLu rat prostatic adenocarcinoma can be prevented *in vivo* by polyamine deprivation," *J. Urol.*, 146:1408-1412, 1991.

Murphy *et al.*, *Cancer*, 78: 809-818, 1996.

Murphy *et al.*, *Prostate*, 26:164-168, 1995.

Nigro, Bull, Boyd, "Importance of the duration of inhibition on intestinal carcinogenesis by difluoromethylornithine in rats," *Cancer Lett.*,(35):183-8, 1987.

Nowels, Homma, Seidenfeld, Oyasu, "Prevention of the inhibitory effects of alpha-diflouromethylornithine on rat urinary bladder carcinogenesis by exogenous putrescine", *Cancer. Biochem. Biophys.*,(8):257-63, 1986.

Partin and Oesterling, *J. Urol.*, 152:1358-1368, 1994.

Pegg, "Polyamine metabolism and its importance in neoplastic growth and a target for chemotherapy," *Cancer Res.*, (48):759-74, 1988.

Pienta, Esper, Zwas, Krzeminski, Flaherty, "Phase II chemoprevention trial of oral fenretinide in patients at risk for adenocarcinoma of the prostate," *Am. J. Clin Oncol*, 20(1):36-39, 1997.

Piironen *et al.*, *Clin. Chem.* 42: 1034-1041, 1996.

Reddy, Nayini, Tokumo, Rigotty, Zang, Kelloff, "Chemoprevention of colon carcinogenesis by concurrent administration of piroxicam, a nonsteroidal anti-inflammatory drug with D,L-α-difluoromethylornithine, and ornithine decarboyxlase inhibitor, in diet," *Cancer Res.*, (50):2562-2568, 1990.

Shantz, Coleman, Pegg, "Expression of an ornithine decarboxylase dominant-negative mutant reverses eukaryotic initiation factor 4E-induced cell transformation," *Cancer Res.*, 56:5136-5140, 1996.

Shantz and Pegg, "Over production of ornithine decarboxylase caused by relief of translational repression is associated with neoplastic transformation," *Cancer Res.*, 54:2313-2316, 1994.

Singh, Kelloff, Reddy, "Intermediate biomarkers of colon cancer, modulation of expression of reas oncogene by chemopreventive agents during azoxymethane induced colon carcinogenesis," *Carcinogenesis*, (14):669-704, 1993.

Silver *et al.*, *Clin. Cancer Res.*, 3: 81-85, 1997.

Singh, Kulkarni, Kelloff, Reddy, "Modulation of azoxymethane-induced mutational activation of *ras* protooncogenes by chemopreventive agents in colon carcinogenesis," *Carcinogenesis*, (15):1317-1323, 1994.

U.S. Patent 4,330,559

U.S. Patent 4,413,141

U.S. Patent 4,499,072

U.S. Patent 4,859,452

U.S. Patent 4,925,835

U.S. Patent 5,002,879

U.S. Patent 5,851,537

U.S. Patent 5,872,104

Verma, "Inhibition of tumor promotion by DL-alpha-difluoromethylornithine, specific irreversible inhibitor of ornithine decarboxylase," *Basic Life Sci.*, 52:195-204, 1990.

Wallon, Shassetz, Cress, Bowden, Gerner, "Polyamine-dependent expression of the matrix metalloproteinase matrilysin in a human colon cancer cell line," *Mol. Carcinog.*, 11:138-144, 1994.

Weeks, Herrmann, Nelson, Slaga, "α- Diflouromethylornithine, an irreversible inhibitor of ornithine decarboxylase, inhibits tumor promoter-induced polyamine accumulation and carcinogenesis in mouse skin," *Proc. Nat'l Acad. Sci. U.S.A.*, (79):6028-32, 1982.

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WO 98/25603